Two-Site Enzyme Immunoassay for Alpha-Fetoprotein in Dried-Blood Samples Collected on Filter Paper

Dean Tsao, Kwong-Jen Hsiao, Jaw-Ching Wu, Chen-Kung Chou, and Shou-Dong Lee

This is a method for measuring alpha-fetoprotein (AFP) in eluates of dried-blood samples on filter paper by use of a simple, sensitive two-site enzyme immunoassay. The spot, 6 mm in diameter (equivalent to about 12 μL of whole blood), is incubated overnight with alkaline phosphatase conjugated to rabbit anti-AFP antibody in a tube containing a polystyrene bead coated with mouse monoclonal antibody to AFP. After the beads are washed, the enzyme activities associated with them are determined colorimetrically, with p-nitrophenyl phosphate as substrate. The measurable range of AFP is from 9 to 900 μg per liter of plasma. AFP in the dried-blood spot as determined by this method correlated well with the AFP value for serum from the same blood sample as determined by radioimmunoassay (r = 0.957, p < 0.001). Preliminary studies in which we used this method with 242 healthy blood donors and 60 patients with hepatocellular carcinoma indicate that it may be suitable for use in mass screening for hepatocellular carcinoma in high-risk populations.

Additional Keyphrases: screening, liver disease, cancer, radioimmunoassay of serum compared, cutoff value

Abelev et al. (1), in 1963, first reported the observation of increased alpha-fetoprotein (AFP) in the serum of mice bearing transplanted hepatocellular carcinoma. In the following year, Tatarinov (2) discovered increased values for AFP in patients with hepatocellular carcinoma. Since then, AFP has become one of the best-studied oncofetal proteins (3). Early diagnosis of hepatocellular carcinoma by AFP measurement has been studied by several researchers using methods that include immunodiffusion (4), RIA (5, 6), Ouchterlony double immunodiffusion, and immunoelectrophoresis (7). These studies show that a very high proportion of AFP-positive cases subsequently proved to be cases of hepatocellular carcinoma. In a few cases, determination of AFP has led to a curative resection of the tumors. Although these studies indicate that the high mortality from liver cancer justifies the screening of the AFP value as one of the possible means of controlling the diseases, the complexities and (or) the high cost of the methods used for AFP measurement have hindered its use in mass screening. This paper describes a sensitive and convenient two-site enzyme immunoassay for AFP in dried-blood samples spotted on filter paper. It may be suitable for use in mass screening for hepatocellular carcinoma in a large high-risk population.

Materials and Methods

Reagents. Alpha-fetoprotein was purified from pooled human cord blood as described by Wu et al. (8). A human AFP standard (72/225) was kindly supplied from the International Laboratory for Biological Standards, World Health Organization, Copenhagen, Denmark, and additional AFP standards were purchased from Dako Corp., Santa Barbara, CA 93103. Alkaline phosphatase was obtained from Boehringer Mannheim GmbH, Mannheim, F.R.G. Bovine serum albumin, p-nitrophenyl phosphate, and diethylaminoethyl-(DEAE)-cellulose were from Sigma Chemical Co., St. Louis, MO 63178. Special filter paper for use in neonatal screening was from Toyo Kagaku Sangyo Co., Tokyo, Japan. Polystyrene beads (6 mm) were from Precision Plastic Co., Chicago, IL 60641. Sepharose 6B, CNBr-activated Sepharose 4B, and Sephadex G-200 were from Pharmacia Fine Chemicals, Upsala, Sweden. All other chemicals were of the highest quality obtainable.
Preparation of rabbit anti-human AFP antibody. Purified human AFP in doses of 100 µg, with Freund's complete adjuvant, was injected into rabbits, followed by three to six additional 100-µg injections, with incomplete adjuvant, at three-week intervals. One month after the final injection, blood was sampled and the serum was separated. The immunoglobulin fraction was prepared from the antisera by column chromatography on DEAE-cellulose, and was further purified by affinity chromatography as described by Rueo et al. (9).

Preparation of monoclonal anti-AFP antibody. The hybridoma cells were prepared by fusion of spleen cells from immunized mice with myeloma cells, SP2, as described by Kennett et al. (10). Monoclonal anti-AFP antibody was obtained from mouse ascites produced by the hybridoma and purified by precipitation with ammonium sulfate and chromatography on DEA-cellulose.

Beads coated with monoclonal anti-AFP antibody. The polystyrene beads were coated with monoclonal anti-AFP antibody by the method of Ziota et al. (11). Polystyrene beads were soaked overnight with monoclonal antibody solution (0.2 g/L in sodium carbonate buffer, 50 mmol/L, pH 9.0) at 4 °C. Then the solution was removed and the beads were rinsed with de-ionized water. Bovine serum albumin solution, 10 g/L in phosphate-buffered isotonic saline, was added to the beads and the mixture was incubated overnight at 4 °C. After being rinsed with phosphate-buffered saline, the beads were stored at 4 °C in phosphate-buffered saline containing, per liter, 5 g of bovine serum albumin and 0.5 g of sodium azide. Under these conditions, approximately 1 µg of antibody was coated onto each bead.

Preparation of rabbit anti-AFP antibody–alkaline phosphatase conjugate. This conjugate was prepared by the one-step glutaraldehyde method described by Ford et al. (12). Reaction was stopped by gel filtration of the conjugate through a column of Sephadex G-200 equilibrated with pH 7.4 buffer containing 0.1 mol of Tris HCl, 0.1 mol of NaCl, 1 mmol of MgCl₂, 0.1 mmol of ZnCl₂, and 0.5 g of NaN₃ per liter. Conjugate separated by this gel filtration was stored in the above buffer containing 10 g of bovine serum albumin per liter.

Preparation of standard AFP in dried-blood spots. Heparinated blood from a normal subject whose concentration of AFP in serum was less than 2 µg/L was used to prepare standard AFP discs as described by Hearn and Hannon (13). Blood was separated into plasma and packed cells. Various quantities of human AFP standard (Dako) were added to the plasma. The AFP concentrations in the plasma were then calibrated against WHO AFP standard (72/225) (14) with use of an AFP enzyme immunoassay kit from Abbott Laboratories, North Chicago, IL 60064. The plasma with calibrated quantities of AFP was mixed with the packed cells to give a hematocrit of 45%. A 25-µL aliquot of blood containing known amounts of AFP was spotted on filter paper and air-dried. These spotted specimens on filter paper were sealed in a plastic bag and stored at −70 °C until use.

Sample collection. Two milliliters of venous blood was sampled from 242 apparently healthy blood donors and from 60 patients with hepatocellular carcinoma. A few drops of the blood were immediately spotted on filter paper and air-dried. Serum was separated from the rest of the blood specimen. Both were kept at −20 °C until use. Results of tests of serum for hepatitis B surface antigen were negative and values for alanine aminotransferase (EC 2.6.1.2) in the serum were within normal limits for all of the 242 healthy blood donors.

There were 55 males and five females with ages ranging from 13 to 72 (median = 60) years in the group of patients with hepatocellular carcinoma.

Samples used for the correlation study were collected from persons presenting for physical check-up and the in-patients.

Assay procedure. A hole puncher was used to punch out a 6-mm sample from the中心 of the dried-blood spot on the filter paper. To each 10 × 45 mm plastic tube containing a 6-mm dried-blood disc (equivalent to about 12 µL of whole blood) obtained from standards or test samples we added 250 µL of pH 7.5 buffer (25 g of bovine serum albumin, 0.1 mol of Tris, and 0.1 mol of NaCl per liter) containing diluted alkaline phosphatase conjugated to anti-AFP antibody. One polystyrene bead coated with monoclonal anti-AFP antibody was then added and the mixtures were gently shaken (120 rpm) overnight (16–20 h) at room temperature.

Beads were washed with four 2-mL portions of de-ionized water—we used this for a Pentax Wash Machine from Abbott Laboratories or a dispenser-aspirator (DA10 MID) from Pharmacia Diagnostics, Uppsala, Sweden—and then transferred to another tube. We added 250 µL of p-nitrophenyl phosphate (2.69 mmol/L) in 1.04 mol/L diethanolamine (pH 10.0) to the washed beads, which were then incubated at 37 °C for 30 min. Then 1 mL of 0.1 mol/L NaOH was added to stop the enzyme reaction. Absorbance at 405 nm was measured in a spectrophotometer (Model 150-20; Hitachi, Tokyo, Japan).

Radioimmunoassay of AFP. Radioimmunoassay of AFP in the serum of the same subject from whom the dried-blood sample on filter paper was prepared was done by the Nuclear Medicine Laboratory of Veterans General Hospital, Taipei, Taiwan, with a commercial RIA kit (Serono Diagnostic SA, Coinsins/VD, Switzerland).

Statistical analysis of results. The "SPSS/PC+" computer program was used for statistical analysis (15).

Results

AFP in concentrations of 9 to 900 µg/Liter of plasma be measured in the 6-mm disc. The sensitivity of the assay can be extended to 5 µg/L, where the signal/noise ratio still significantly exceeds 1.4. Typically, a dried-blood disc containing no detectable AFP gives an absorbance of 0.02 A.

As shown in Figure 1, the intensity of the color reaction started to decline in the dried-blood samples at AFP concentrations exceeding 50 mg/L. However, even for concentrations of AFP up to 3 g/L the color intensity was still greater than that for 900 µg/L.

As shown in Table 1, the within-assay coefficients of variation (CVs) were 3.7 to 9.6%, the between-assay CVs 10.2 to 10.6%. Analytical recovery of AFP ranged from 106 to 110% for standards added to blood in the range of 9 to 900 µg per liter of plasma. AFP in the dried-blood samples is stable for at least a month at room temperature.

We compared AFP concentrations measured in 94 dried-blood samples by the present assay with those determined by RIA in paired serum from the same subjects (Figure 2). There was a good correlation: $r = 0.957$, $p < 0.001$. The slope and intercept of the linear-regression equation were not statistically different from 1 and 0, respectively. The comparison of the data was calculated according to the IFCC recommends (16).

For the 242 cases of healthy blood donors and 60 known cases of hepatocellular carcinoma, there was an excellent agreement between results by the present assay and those.
The final determination for serum of the same subject by radioimmunoassay (Table 2). About 72% of the patients with hepatocellular carcinoma were found to have increased AFP concentrations (>20 µg/L) by both methods; none of the 242 blood donors had an AFP value >20 µg/L.

**Discussion**

Mass-screening programs for detecting hepatocellular carcinoma among high-risk populations by the AFP test have been shown by several laboratories (4-7) to be useful in early diagnosis of the disease. However, the techniques used for AFP assay were less than satisfactory because of the difficulty of collecting and transporting blood samples and the complexity and (or) insensitivity of the method.

We adapted the dried-blood spot technique, a method widely used in neonatal screening for congenital metabolic diseases (17), in our assay system, to simplify the sample collection and transportation processes. This system is convenient for use in mass screening programs, especially in rural areas. Although data are not shown here, AFP in the dried-blood spot is very stable: we detected no significant decrease of AFP activity in the spot even after the filter paper was incubated at 37 °C for a month. Thus dried-blood spot samples can validly be transported from a remote area to a centralized laboratory. This extraordinary stability of AFP in dry form also has been reported by Wu and Knight (18).

Second, with a combination of monoclonal anti-AFP antibody coated to a solid phase and an enzyme-conjugated polyclonal anti-AFP antibody, we were able to construct a two-site enzyme immunoassay. Under the assay conditions, test samples can be incubated with both antibodies simultaneously, and only a single washing step is needed before the color reaction. This minimizes manipulation and shortens the hands-on time required for the assay.

One must be careful of a "hook" effect in a two-site immunoassay if concentrations of AFP are high, as previously reported by Nomura et al. (19). We examined our present assay for concentrations of AFP as high as 3 g/L and found that the intensity of the color reaction still exceeded that of the maximum concentration of AFP (900 µg/L) used in our standards. This may be due to the slow release of AFP from the dried-blood spot into the surrounding reaction mixture. The highest AFP concentration that we have encountered in the serum of 60 patients with hepatocellular carcinoma was 450 mg/L, so it is unlikely that the "hook" effect will be a concern.

The reliability of the present method is shown by the good correlation between AFP values for the discs and, by RIA, for serum from the same subject. Also, results were almost identical for healthy blood donors and also for patients with

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**Table 1. Precision of the Present Assay**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AFP concn, µg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Within assay</strong> (n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>18.41</td>
<td>1.76</td>
<td>9.58</td>
</tr>
<tr>
<td>B</td>
<td>77.91</td>
<td>2.88</td>
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</tr>
<tr>
<td>Mean</td>
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<tr>
<td><strong>Between assay</strong> (n = 10)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>19.66</td>
<td>2.01</td>
<td>10.24</td>
</tr>
<tr>
<td>D</td>
<td>77.91</td>
<td>8.24</td>
<td>10.58</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>10.41</td>
</tr>
</tbody>
</table>

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**Table 2. Summary of Determinations of AFP in Dried-Blood Spots and in Paired Sera from Healthy Persons and from Patients with Hepatocellular Carcinoma**

<table>
<thead>
<tr>
<th>AFP concn, µg/L</th>
<th>No. of healthy persons</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Spot</td>
</tr>
<tr>
<td>&lt;20</td>
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<td>242</td>
</tr>
<tr>
<td>20–299.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>300–899.9</td>
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<td>0</td>
</tr>
<tr>
<td>≥900</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>242</td>
<td>242</td>
</tr>
</tbody>
</table>

*AFP determined by present assay in spots, by RIA in serum.*
hepatocellular carcinoma by the two methods (Table 2). No normal subject showed an AFP value >20 μg/L, but more than 70% of the patients with hepatocellular carcinoma showed a higher AFP value. The increased AFP concentrations in the blood of liver-cancer patients suggests that screening by AFP test may offer the best hope of early detection of this cancer (20). Of course, AFP also is increased in serum of patients with other liver diseases (3). The best cutoff value to use for AFP in mass screening for hepatocellular carcinoma remains to be determined.

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References